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Purification of Human Red Cell Acetylcholinesterase by Electrophoresis, Ultracentrifugation and Gradient Extraction

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Introduction

The solubilization and purification of the acetylcholinesterase in human red blood cells approximately 250-fold have been reported (1). Additional purification has been accomplished by the use of electrophoresis on paper, by the use of the ultracentrifuge, and by ammonium sulfate–gradient extraction. The application of these methods reveals some of the properties of the cholinesterase preparation.

Methods

The assay of the cholinesterase, and the determination of protein have been described (1).

Electrophoresis on Paper

The procedure of Kunkel and Tiselius (2) is followed in which the paper is placed between two glass plates with the ends of the paper dipping into electrode vessels. For the small-scale, orienting experiments, two papers in layers were used. On one, the protein distribution was determined by staining with bromophenol blue; on the other, the esterase was eluted with water from 1.0-in. wide strips and determined by assay. In experiments at pH 8.5, all components moved toward the positive electrode with the bulk of the cholinesterase activity occupying an intermediate position on the paper at the end of an 18-hr. run. By eluting the esterase-rich fraction, a 2- to 3-fold increase in purity was obtained (1). The scale of the electrophoresis is conveniently increased by increasing the size and the number of the papers so that 400 mg. of protein can be handled, and the procedure can be used as a step in the purification of the cholinesterase. This is done

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as follows. Five sheets of Whatman 3 MM paper,[2] 5.5 × 22.5 in., are used in layers. A flap (2) is cut in the papers near one end, with the cuts sloping inward. The papers are wet with buffer (phosphate–borate, pH 8.6, ionic strength 0.07). The edges of the flap are raised, dried partially with filter paper, and the cholinesterase (400 mg. in 3.0 ml. buffer) placed uniformly on the cut edges. The flap is then pressed back in place, and the ends of the paper are dipped into buffer. The color of the cholinesterase preparations makes possible a visible regulation of the uptake of buffer. Electrophoresis is carried out at 7° for 18 hr. at 450 v. At the end of this period the paper is cut into three sections with cuts usually 1 in. behind the starting point, and 4.0, 9.0, and 15.0 in. from the starting point. The three portions of paper are each extracted successively three times with 50-ml. portions of water. With each extraction the papers are squeezed dry on a Büchner funnel with a coarse sintered-glass plate. Usually 90–100% of the esterase activity is recovered.

Use of Ultracentrifuge

The type L Spinco ultracentrifuge is used with No. 40 rotor which holds 12–13.5-ml. size tubes. Plastic tubes are used with metal tops which can be entered through a center opening. Samples are removed from the tubes with a mounted syringe and needle which is moved mechanically to the desired level in the centrifuge tube. To prevent stir-back, when the centrifuge is stopped, of the concentration gradients obtained by centrifugation, a sucrose density gradient is used (3). To obtain this, 5 ml. of 1% cholinesterase solution containing 10% sucrose is placed in the bottom of the centrifuge tube, and 6.5 ml. of 1% esterase containing no sucrose is layered on the top. The gradient at the sucrose boundary is broadened by stirring lightly with a rod. The ultracentrifuge is run for 6–7 hr. at 40,000 r.p.m. (140,000 × g). At the end of the run the top 9.5 ml. are withdrawn with a needle and syringe; the bottom 2.0 ml. are decanted. A pellet of sediment is dissolved in 2.0 ml. of ethanolamine buffer, pH 9.6. Over 50% of the esterase is found in the bottom fraction with the purity twofold or greater than that of the starting material.

Gradient Extraction

This procedure is based on a method described by Zahn and Stahl (4). Its application to the separation of purified proteins has been studied (5). In this method a mixture of proteins is precipitated with a salt, and the precipitate, on an inert carrier, is extracted with the same solution continuously diluted with water. Pure proteins, when extracted in this manner, appear in a narrow zone of the extract with a symmetrical distribution of the protein about a maximum (5). In the cholinesterase experiments, approximately 150 mg. of solids is precipitated with 2.4 M ammonium sulfate, and the precipitate is distributed in a column of 7 g. of Hyflo. The precipitate is extracted with ammonium sulfate diluted so that with each 50 ml. of extractant the concentration decreases linearly about 0.5 M. Ten-milliliter samples are collected, and protein and esterase activity in each are determined.

[2] Mention of products in this paper does not imply endorsement or recommendation by the Department of Agriculture over similar products not mentioned.

Results

Electrophoresis on Paper

The results obtained in a typical electrophoresis experiment are shown in Table I. Data are shown for the summation of the three extractions obtained of each fraction. In general, 70.0% of the esterase is obtained in the first extract, 22.0% in the second, and 8.0% in the third. The increase in purity is two-fold over that of the starting material, our standard preparation (1). Sixty-one per cent of the esterase is found in the fraction of intermediate mobility. The "hemoglobin" is enriched about 10% in the more rapidly moving fraction.

Ultracentrifuge

The results with the ultracentrifuge are illustrated in Table I. The increase in purity in the bottom fraction is approximately two-fold; this fraction contains over 60% of the esterase. In this instance the "hemoglobin" is enriched in this fraction also. The pellet fraction shows some opalescence, as does the slow fraction in the electrophoresis experiment. The association of the cholinesterase activity with material of high molecular weight is also supported by ultrafiltration experiments. In experiments with a 0.2% solution of the standard preparation, 92% of the cholinesterase activity is retained by a "coarse" ultrafilter (Schleicher and Schuell type II). More than 30% of the total protein, however, is in

TABLE I

Distribution of Esterase and Protein in Paper Electrophoresis and Ultracentrifuge Fractions

Fraction	Activity units/ mg. protein	Activity units %	Protein %	"Hemoglobin"[a] *% of total protein*
		Electrophoresis		
Starting material	950	100.	100.	9.7
Fast	381	14.5	36.6	10.8
Intermediate	1800	61.0	32.2	8.0
Slow	748	24.5	31.2	7.25
		Ultracentrifuge		
Starting material	1370	100.	100.	10.2
Top	625	20.	45.	10.
Bottom	2690	60.	30.5	11.4
Pellet	1115	20.	24.5	8.5

[a] This is expressed as hemoglobin but is not hemoglobin (see *Discussion*), and the nature of the heme has not been clarified.

the filtrate. With the more highly purified preparations, 98% of the activity is retained by the "coarse" ultrafilter.

Gradient Extraction

When our standard preparation (1) is precipitated with ammonium sulfate and then extracted with diluted solvent, two main fractions are obtained. The first is brought into solution with 1.9 *M* ammonium sulfate, the second when the concentration has dropped to 1.0 *M*. The esterase of greatest purity (about two-fold increase) is found in the 1.9 *M* fraction, with esterase of enhanced purity on the upslope of the 1.0 *M* extraction curve. The esterase-rich fractions from the electrophoresis and ultracentrifuge experiments, however, when subjected to gradient extraction, contain almost exclusively the second, less soluble fraction, with the protein distribution in the extract indicative of a pure protein.

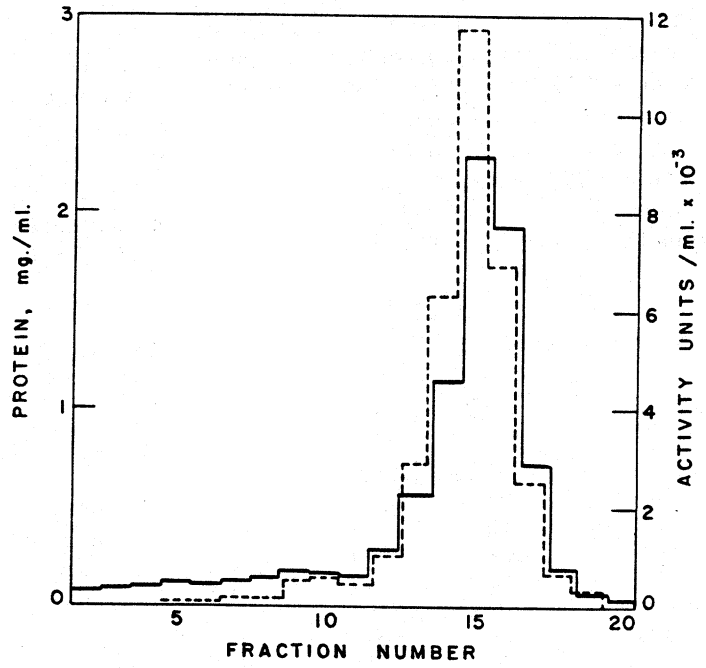

FIG. 1. Solvent–gradient extraction of a purified cholinesterase preparation. The protein concentration in successive fractions of the extract is given by the solid line, the cholinesterase activity by the dashed line.

The results obtained with one of these preparations is shown in Fig. 1. The moderate increase in purity obtained in this instance occurs in the fractions on the upslope of the extraction curve. "Hemoglobin" is found in all of the fractions, with a slightly enhanced concentration in the 1.0 *M* fraction.

Discussion

The purification of cholinesterase is summarized in Table II. Steps 1–5 recapitulate briefly the preparation of the material described previously (1), termed the standard preparation. Steps 6 and 7 indicate the results obtained with the methods described in the present report. Changing the order in which steps 6 and 7 are used, or the repeated use of either method, leads to about the same result.

Although electrophoresis, ultracentrifugation, and gradient extraction are all means for furthering the purification of cholinesterase, their use has not led to the complete purification of the cholinesterase. In most cases increase in purification on repetition of the procedures diminishes rapidly at a level of purity of 3000 units/mg. protein. This leveling-off apparently coincides with the separation of a major protein with which the cholinesterase is tenaciously associated.

Most of the purification was carried out with electrophoresis and ultracentrifugation. Gradient extraction was used batchwise to raise the level of some of the standard preparations. The product obtained with these methods appears to be a single protein. The cholinesterase is associated with this protein but, since it may constitute less than 5% of the total protein (1), it is not apparent as a separate entity with physicochemical tests. The apparent homogeneity of this fraction revealed by gradient

TABLE II

Purification of the Acetylcholinesterase in Human Red Blood Cells

Human red blood cells

1. Hemolysis of cells; washing of stromata.
2. Stromata precipitated with cadmium acetate; further washing.
3. Esterase dissociated from stromata with Tween 20.
4. Purification with calcium phosphate and clarification with Filter-Cel.
5. Dried in frozen state; lipide and remaining Tween removed with acetone, ethanol, and ethyl ether. Dry, stable powder: 1100 activity units/mg. protein; yield: 20.0% of starting activity.
6. Electrophoresis on paper. 2000 units/mg. protein; yield: 12.0%.
7. Ultracentrifuge. 3000–4000 units/mg. protein; yield: 7.0%.

extraction as shown in Fig. 1 was confirmed by free electrophoresis. The esterase-rich fraction in Veronal buffer, pH 8.5, ionic strength 0.1, migrated with a mobility of 4.2×10^{-5} sq. cm./v./sec., with a single, quite symmetrical concentration–gradient peak as shown in Fig. 2. For comparison, the pattern obtained with our standard preparation is shown. This illustrates the selectivity of the methods used in the present study. Some specific means of dissociating the cholinesterase from the major protein must be found to make further purification of the cholinesterase by the present means practical. Continuous increases in purity (10–20%) on repetition of these methods, together with other evidence, indicate that the major protein is not the cholinesterase protein, but increases of this level are impractical for attaining complete purification. The cholinesterase in these preparations appears to be less stable than in the standard preparation.

The total cholinesterase activity is symmetrically, but broadly, distributed during electrophoresis on paper. A pure protein, like β-lactoglobulin for example, is distributed over about 5 cm. of paper in the course of an 18-hr. experiment. Cholinesterase, on the other hand, is distributed over 20 cm., with the peak 10–15 cm. from the starting point. This may reflect a slow dissociation from another protein rather than an electrical inhomogeneity of the cholinesterase molecule. There is considerable evidence that the "hemoglobin" color is not hemoglobin. On electrophoresis it moves toward the positive pole at pH 6, whereas hemoglobin moves toward the negative pole. The esterase preparations have a featureless absorption between 500 and 600 mμ; however, when reduced with hydrosulfite, a typical hemochromogen absorption is obtained with

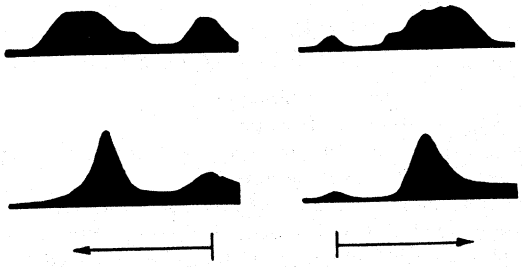

FIG. 2. Electrophoresis of cholinesterase preparations at pH 8.5. The upper set of patterns was obtained with the standard preparation; the lower set was obtained with a preparation purified by electrophoresis on paper and ultracentrifugation. The patterns were obtained after electrophoresis for 190 min.

a minor peak at 525 mμ, and a major peak at 555 mμ, dissimilar from either hemoglobin or denatured hemoglobin. The heme apparently is none of the usual heme-containing enzymes; tests for peroxidase and catalase are negative and cytochrome c is excluded by the electrophoretic results. Free heme is a possibility, but if it is in the free form it is very tenaciously associated with protein. Use of adsorbents, etc. has failed to remove the heme, as well as use of the recently described (6) removal of hemin as aggregates at pH 4.75. It is of interest that of a number of species studied (7), the stromata from human red blood cells contain the most "hemoglobin" that resists removal by washing.

Summary

Purification of human red cell cholinesterase by means of electrophoresis on paper, ultracentrifugation, and gradient exctraction is described. By these means a protein is isolated that appears to be homogeneous in gradient extraction and free electrophoresis; however, reasons are given for believing that this is not the cholinesterase protein but a major protein with which the cholinesterase is tenaciously associated.

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